

# Effect of *Sorbus commixta* on the invasion and migration of human hepatocellular carcinoma Hep3B cells

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**Abstract.** Tumor metastasis is a main cause of cancer-related morbidity and mortality. Thus, a number of medicinal herbs and phytochemicals have been investigated as possible candidates for the inhibition of cancer metastasis. *Sorbus commixta* Hedl. (SC) is a traditional medicinal plant used in the treatment of inflammatory diseases, as it has antioxidant, anti-inflammatory, anti-atherosclerotic and anti-hepatotoxic activities. In this study, we demonstrate that the water extract of SC exerts inhibitory effect on the invasion and migration of hepatocellular carcinoma Hep3B cells. The activity and expression of matrix metalloproteinase (MMP)-9, which is responsible for the invasion of cancer cells, was decreased by SC treatment. The invasive and migratory potentials of the Hep3B cells were also decreased, as evidence by *in vitro* assay using the Boyden chamber system. In addition, the expression of the chemokine receptors, C-X-C chemokine receptor type 4 (CXCR4) and C-X-C chemokine receptor type 6 (CXCR6), were inhibited by SC in Hep3B cells. Furthermore, actin fiber organization was markedly suppressed by SC treatment. Taken together, the findings of this study suggest for the first time, to the best of our knowledge, that SC suppresses the invasion and migration of highly metastatic Hep3B cells.

## Introduction

Frequent metastasis is the main cause of cancer-related recurrence and mortality, including hepatocellular carcinoma (HCC) (1,2).

The metastatic processes of cancer consists of complex steps, i.e., the invasion of cancer into tissues around the primary sites, entry into vascular or lymphatic systems, survival in the bloodstream, adhesion onto vascular endothelial cells in small vessels around new tissues, extravasation from the circulation into surrounding tissues, and finally growth, to form migrated tumors in new sites (3-5). Thus, in order to prevent and treat cancer metastasis, researchers are trying to understand the molecular mechanisms responsible for cancer metastasis and to develop novel and effective anti-metastatic drug candidates (4,6).

A number of medicinal herbs and phytochemicals have been reported as potent candidates for anti-metastatic drugs (7-9). *Sorbus commixta* Hedl. (Rosaceae) (SC) is a traditional medicinal plant used in the treatment of inflammatory diseases, such as asthma, bronchitis, gastritis and edema (10,11). Previously, SC was reported to have antioxidant (12,13), anti-inflammatory (11), anti-atherosclerotic (14-16) and anti-hepatotoxic activities (17). In addition, SC has been shown to inhibit the expression of matrix metalloproteinase (MMP)-1 (12) and tyrosine phosphatases activity (18), which are associated with the migration and development of cancer (19-21). However, the inhibitory effects of SC on cancer metastasis have not been reported to date, at least to the best of our knowledge.

In this study, we demonstrate that the water extract of SC inhibits the invasion and migration of the highly metastatic HCC cell line, Hep3B. The underlying mechanisms of the anti-invasive and anti-migratory effects of SC are associated with the inhibition of MMP-9 activity and expression, chemokine receptor expression, and actin filament arrangement. To the best of our knowledge, this is the first study to demonstrate that SC exerts anti-invasive and anti-migratory effects on HCC cells.

## Materials and methods

**Materials.** Antibody to MMP-9 (AB18916) was supplied by Calbiochem (San Diego, CA, USA). Antibodies against phospho-extracellular signal regulated kinase (ERK; sc-7383), ERK2 (sc-154), p38 (sc-535), c-jun N-terminal kinase (JNK; sc-7345), p65 subunit of NF- $\kappa$ B (p65; sc-50350), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-32233) were provided

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by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for phospho-p38 (#9211), phospho-JNK (#9255), and inhibitor of NF- $\kappa$ B (IkB; #9242) were supplied by Cell Signaling Technology (Danvers, MA, USA). Antibody against  $\beta$ -actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies for goat anti-mouse IgG (NCI1430KR) and goat anti-rabbit IgG (NCI1460KR) were provided by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All chemicals and reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lupenone, lupeol and prunetin, were obtained from Sigma-Aldrich unless indicated otherwise.

**Extraction of SC.** The stem and cortex of SC, which were grown and collected in Gyeongsangbuk-do province in Korea in 2012, were purchased from Omniherb Co. (Daegu, Korea) and authenticated by botanical expert analysis by the company. A voucher specimen is kept at the School of Korean Medicine, Pusan National University, Yangsan, Korea. The air-dried stem and cortex of SC (50 g) was cut and extracted with distilled water (1 l) at 100°C for 4 h. The extract was filtered with Whatman paper (6  $\mu$ m; Whatman PLC, Kent, UK) and concentrated using a rotary evaporator (Eyela, Tokyo, Japan). Subsequently, the extract was lyophilized using a freeze dryer (Labconco Corporation, Kansas city, MO, USA) to yield 3.1 g of powder (abbreviated as SC). The powder was dissolved in DMSO for stock solution (100 mg/ml) and diluted with culture medium prior to use in the experiments.

**Cell culture and determination of cell viability.** Hep3B, HepG2 HCC cells, Chang liver cells and HCT116 colon cancer cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with L-glutamine (200 mg/l), 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C, 5% CO<sub>2</sub> prior to the experiments.

The cytotoxicity caused by SC was evaluated using an MTT assay. Briefly, the Hep3B cells were incubated in 24-well plates with the indicated concentrations of SC for 24 h. Subsequently, MTT solution (2.0 mg/ml) was added to each well containing the cells. Following 4 h of incubation at 37°C and 5% CO<sub>2</sub> in a cell culture incubator, the conditioned medium was removed, and formazan crystals formed in living cells were estimated by measuring the absorbance at 540 nm using a microplate reader (SpectraMax M2; Molecular Devices LLC, Sunnyvale, CA, USA). The percentages of living cells were calculated against the control cells by dividing each of the absorbance values by the average of that from the control sample without treatment with SC.

**Gelatin zymography assay.** Gelatin zymography assay for detecting MMP-9 activity was performed according to a previously described method (22). For *in vitro* gelatinase activity assays, culture medium from the Hep3B cells was used for the source of enzyme. The cells were cultured in serum-free medium for 24 h and equal amounts of medium were resuspended in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 0.00625% (w/v) bromophenol blue,

and then loaded without boiling in a 7.5% acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin. Electrophoresis was performed at a voltage of 100 V at 4°C. Following electrophoresis, the gel was soaked twice in 0.25% Triton X-100 for 30 min at room temperature and washed in pure distilled water. The gel slab was cut into slices corresponding to the lanes, and then placed into different tanks containing the indicated concentrations of SC, freshly solubilized in Tris-buffered saline. The gels were soaked in incubation buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM CaCl<sub>2</sub> and 0.02% Brij 58. The gels were incubated at 37°C for 18 h and then stained for 30 min in 0.1% (w/v) Coomassie blue R-250 (Sigma-Aldrich) solubilized in 30% methanol and 10% acetic acid.

For detecting gelatinase activity in the cell culture, Hep3B cells were grown in serum-free culture medium with the indicated concentrations of SC for 24 h. The amount of secreted protein in the conditioned medium was estimated by cell numbers. Culture media were prepared in sample buffer mentioned above without boiling. The samples were loaded in an acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin, and then electrophoresed at 4°C. The gels were washed twice in 0.25% Triton X-100 for 30 min at room temperature and incubated at 37°C in incubation buffer same as the one mentioned above. Following incubation at 37°C for 18 h, the gels were stained for 30 min in Coomassie blue R-250 staining solution. The stained gel was photographed and bands corresponding to the activity of MMP-9 were quantified using ImageJ software (NIH, Bethesda, MD, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR).** To measure the changes in the mRNA expression levels related to tumor metastasis, Hep3B cells were treated with the indicated concentrations of SC (0, 50, 100  $\mu$ g/ml) in serum-free DMEM, and incubated for 24 h. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA; Bio-Rad Laboratories, Inc., Hercules, CA, USA). An equal amount of total RNA was used for the synthesis of cDNA using oligo-dT primer and AccuPower RT-PreMix (Bioneer Co., Daejeon, Korea). The cDNA was amplified by PCR with the following primers: MMP-9, 5'-AGTCCGGCA GACAATCCTTGCA-3' (sense) and 5'-ATCCACGCGAATG ACGCTCTGG-3' (antisense); C-X-C chemokine receptor CXCR4, 5'-AGCCTGTGGATGGTGGTGTTC-3' (sense) and 5'-CCTTGCTTGATGACTCCCAAAAG-3' (antisense); CXCR6, 5'-TACGATGGGCACTACGAGGGAG-3' (sense) and 5'-GCAAAGAAACCAACAGGGAGACCAC-3' (antisense); GAPDH, 5'-GGAGCCAAAAGGGTCATCAT-3' (sense) and 5'-GTGATGGCATGGACTGTGGT-3' (antisense). The amplification of the PCR reactions was performed with AccuPower PCR-PreMix (Bioneer Co.), under the following conditions: an initial denaturation at 95°C for 5 min followed by 30 cycles (for MMP-9 and GAPDH) or, 35 cycles (for CXCR4 and CXCR6) of denaturation for 40 sec at 95°C, annealing for 40 sec at 60°C (for MMP-9 and GAPDH) or, 63°C (for CXCR4 and CXCR6) and extension for 50 sec at 72°C with a final extension for 10 min at 72°C. Amplified PCR products were separated on 1.0% agarose gels and visualized under UV light. Images were captured using the GelDoc-It TS imaging system (UVP, Upland, CA, USA).

**Western blot analysis.** To examine the effect of SC on the regulation of protein expression related to tumor metastasis, we treated the cells with the indicated concentrations of SC (0, 50, 100  $\mu\text{g/ml}$ ) in serum-free DMEM, followed by incubation for 24 h. Total proteins were extracted from the cells using RIPA buffer (Cell Signaling Technology) containing 1 mM phenylmethylsulfonyl fluoride. The amounts of proteins were estimated using the Quick Start™ Bradford Protein assay (Bio-Rad Laboratories, Inc.). An equal amount (20  $\mu\text{g}$ ) of protein from each sample was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was transferred by electrophoresis onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Uppsala, Sweden). The membranes were blocked for 1 h with 5% non-fat dry milk at room temperature and incubated with primary antibodies specific for the target protein at 4°C overnight. The membranes were washed twice, and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase. The specific bands of the proteins of interest were detected using ECL Plus (GE Healthcare).

**Transwell invasion and migration assay.** The filter inserts (8  $\mu\text{m}$  pore size) that fit into 24-well invasion chambers (Becton-Dickinson, Franklin Lakes, NJ, USA) were used for invasion and migration assays. For the invasion assay, the upper sides of the filters were coated with 100  $\mu\text{l}$  of 0.2% collagen-fragmented gelatin. Serum-free DMEM with or without SC (indicated concentrations) was added to the upper compartment of the chamber. In the lower part of the chamber, DMEM supplemented with 10% FBS was added. The chambers were incubated at 37°C for 24 h in a 5%  $\text{CO}_2$  atmosphere. For the migration assay, serum-free DMEM with or without SC (indicated concentrations) was added to the upper part of the chamber. To the lower part of chamber, DMEM supplemented with 10% FBS was added to make a gradient of chemoattractive factors. The chambers were incubated at 37°C for 24 h in a 5%  $\text{CO}_2$  atmosphere. Following incubation, the filter inserts from both of invasion or migration assay were removed from the chamber well, and the cells on the upper side of the filter membranes were removed using cotton swabs. The membranes were fixed, stained with hematoxylin and eosin (HHS16; Sigma-Aldrich), and mounted on microscope slide glasses. The cells located on the underside of the filters were counted for invaded or migrated cells.

**F-actin staining and fluorescence microscopy.** The Hep3B cells were seeded on sterilized 12 mm  $\pi$  rounded cover slides in 24-well culture plates, and treated with or without SC (indicated concentrations) for 24 h. The cells were washed with phosphate-buffered saline (PBS) and fixed in 3.7% formalin for 10 min. The cells were washed with PBS, and then permeabilized with 0.1% Triton X-100 in PBS. For actin staining, the cells were washed with PBS, and then incubated with PBS including 1 U of Texas Red-X phalloidin (Invitrogen) for 30 min at room temperature. The cells were washed with PBS again, and were then analyzed using a confocal microscope (Axio Observer Z1; Carl Zeiss, Oberkochen, Germany).

**Statistical analysis.** The values from the cell viability, gelatin zymography, invasion and migration assays were

calculated by the percentage of control cells and expressed as the means  $\pm$  SD. The differences between the mean values compared with the control group were evaluated by one-way analysis of variance (ANOVA) with a post hoc Dunnett's comparison. The minimum level of significance was set at a P-value of 0.05 for all the analyses. All the experiments were independently carried out at least 3 times.

## Results

**Cytotoxic effects of SC on Hep3B cells.** Initially, we examined the cytotoxic effects of SC on several cell lines, such as Hep3B, HepG2, Chang and HCT116 cell, in order to determine the optimal concentration for use in subsequent experiments. SC exerted a significant cytotoxic effect on the Hep3B cells at 250  $\mu\text{g/ml}$ . In addition, SC significantly inhibited the growth of HepG2 HCC cells from the concentration of 200  $\mu\text{g/ml}$ , and that of the HCT116 colorectal cancer cells from the concentration of 100  $\mu\text{g/ml}$ . In addition, SC significantly attenuated the growth of Chang cells from the concentration of 500  $\mu\text{g/ml}$ . SC (250  $\mu\text{g/ml}$ ) suppressed the growth of Chang cells, although no significant differences were observed (Fig. 1). Among the liver cancer cell lines, the Hep3B cells exhibit the most potent invasive and migratory potential (23). Thus, we examined the inhibitory effects of SC on the invasion and migration of liver cancer cells using the Hep3B cells and SC at up to a concentration of 100  $\mu\text{g/ml}$ .

**SC inhibits the activity and expression of MMP-9.** We then examined the effects of SC on the activity and expression of MMP-9. The results from *in vitro* gelatin zymography assay revealed that SC significantly inhibited enzyme activity from the concentration of 50  $\mu\text{g/ml}$  (Fig. 2A). In addition, when SC was added to the cell culture medium of Hep3B cells, the activity of MMP-9 was also decreased (Fig. 2B). These results suggested that the expression level of MMP-9 was reduced by SC treatment. Thus, we confirmed the expression of MMP-9 using RT-PCR and western blot analysis. The results revealed that the mRNA and protein levels of MMP-9 were decreased at the concentration of 100  $\mu\text{g/ml}$  (Fig. 2C and D).

**SC decreases the invasive and migratory potential of Hep3B cells.** As MMP-9 is a major enzyme responsible for the destruction of extracellular matrix, an initial step of cancer metastasis (24,25), we examined the effects of SC on the invasive property of Hep3B cells. The results from invasion assay using collagen-degraded gelatin as a substrate demonstrated that SC significantly reduced the numbers of invading Hep3B cells (Fig. 3). We then evaluated the effects of SC on the migratory activity of Hep3B cells using Boyden chamber assay. FBS was added to the lower chamber for making a chemoattractant gradient. The results revealed that SC significantly inhibited the migratory ability of the Hep3B cells in a dose-dependent manner (Fig. 4).

**SC decreases the expression of chemokine receptors and arrangement of actin filament.** Chemokine receptors expressed on the surface of cancer cells, particularly CXCR4 and CXCR6, play important roles in tumor metastasis (26,27). Thus, we examined the expression of CXCR4 and CXCR6 in Hep3B cells.

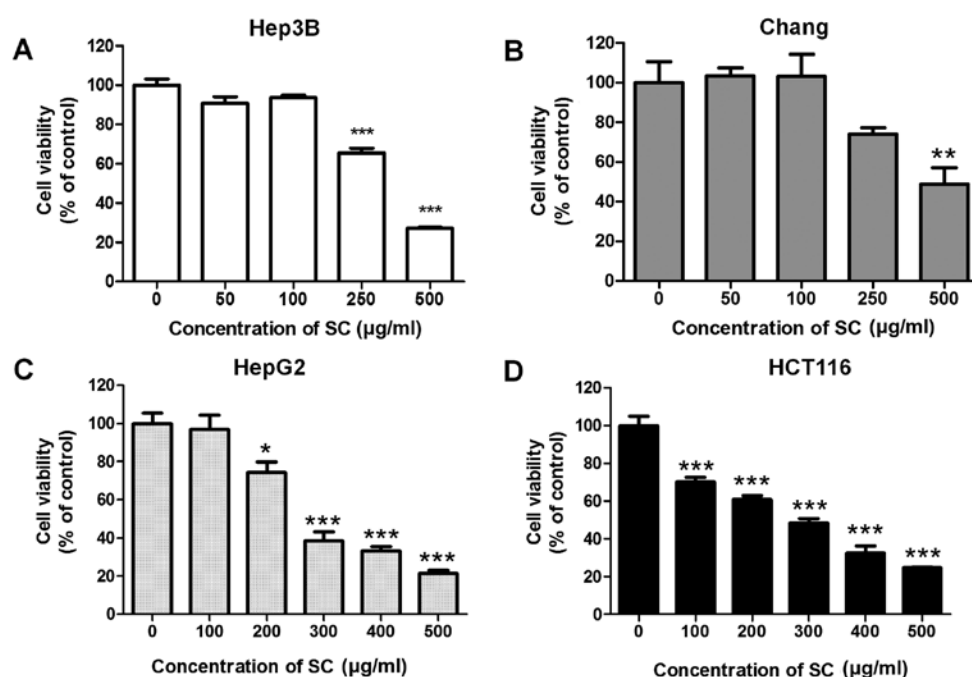


Figure 1. Cytotoxicity of *Sorbus commixta* (SC) on Hep3B, HepG2, Chang and HCT116 cells. (A-D) The cells were treated with the indicated concentrations of SC for 24 h. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results from 3 independent experiments are shown as the means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to the control.

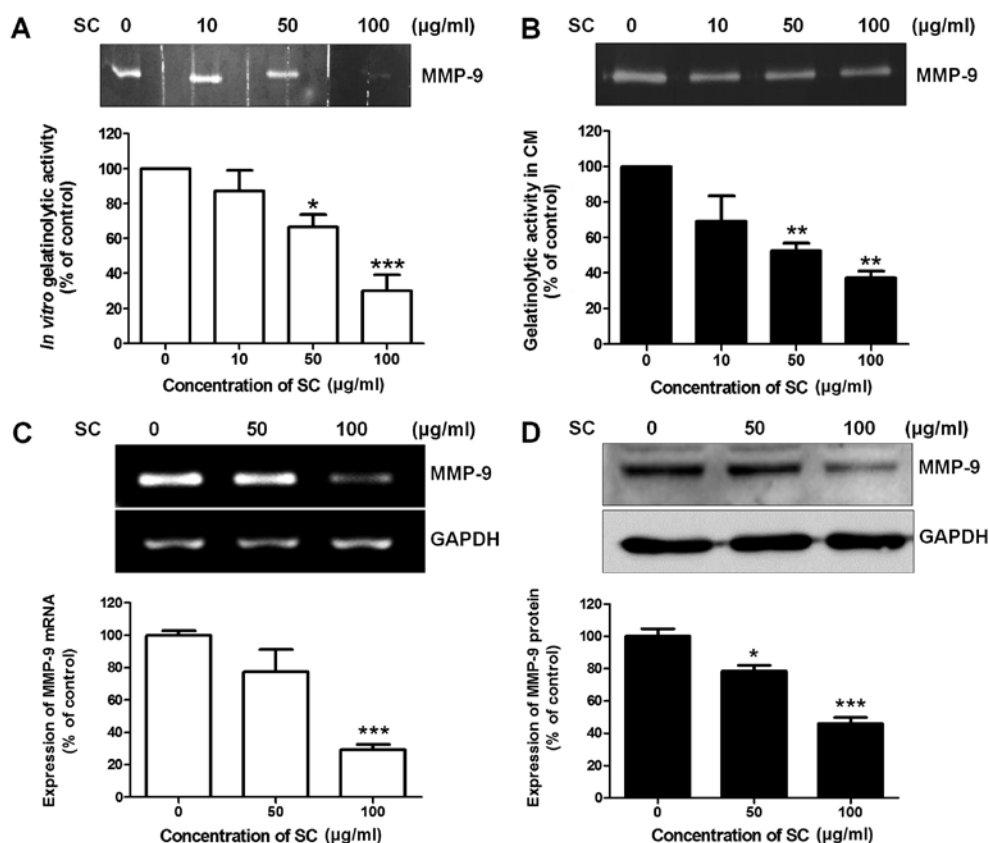


Figure 2. Inhibitory effect of *Sorbus commixta* (SC) on the activity and expression of MMP-9 in Hep3B cells. (A) *In vitro* gelatin zymography assay was performed using the culture medium of Hep3B cells as a source of enzyme. Indicated concentrations of SC were added into incubation buffer. Densitometric results of the bands are presented as the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$  compared to the control. (B) Hep3B cells were cultured in medium containing the indicated concentrations of SC for 24 h. The same amounts of the culture medium were analyzed by zymography for gelatinolytic activity. Densitometric data of the bands were shown as the means  $\pm$  SD of 3 independent experiments. \*\* $P < 0.01$  compared to the control. (C and D) Hep3B cells were treated with the indicated concentrations of SC for 24 h. RT-PCR and western blot analysis were performed to evaluate the expression of matrix metalloproteinase (MMP)-9. Densitometric results of the bands are presented as the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$  compared to the control.

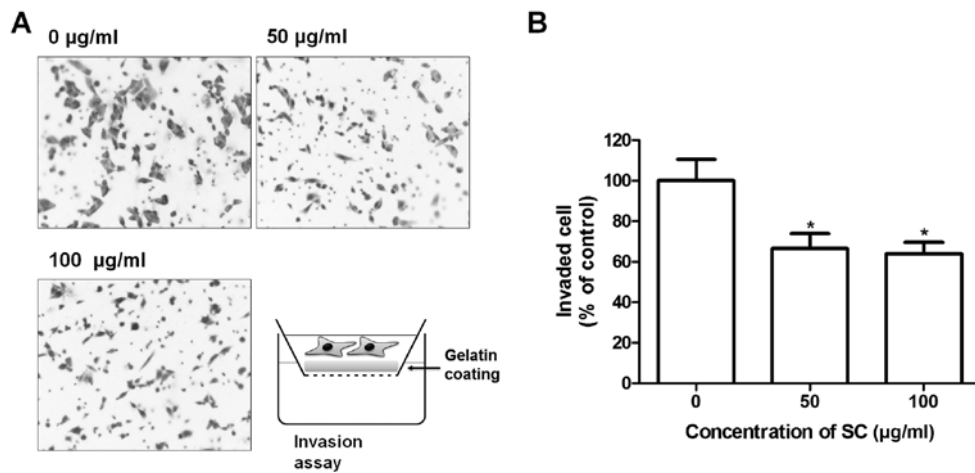


Figure 3. Inhibitory effect of *Sorbus commixta* (SC) on the invasion of Hep3B cells. Hep3B cells were seeded in a gelatin-coated upper chamber and incubated in medium containing the indicated concentrations of SC for 24 h. The lower part of the membrane was stained with hematoxylin and eosin (H&E). (A) Representative images and (B) counted results are shown. The data demonstrated as the means  $\pm$  SD of 3 independent measurements. \* $P < 0.05$  compared to the control.

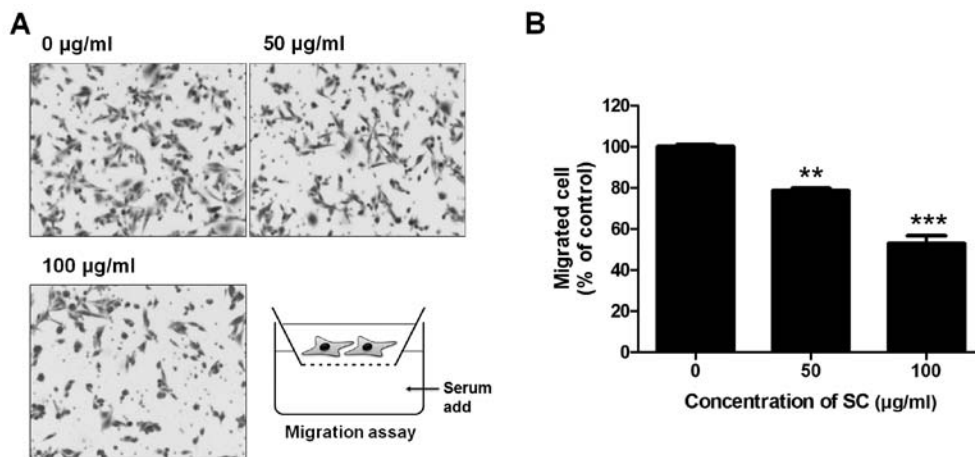


Figure 4. Inhibitory effect of *Sorbus commixta* (SC) on the migration of Hep3B cells. Hep3B cells were seeded in the upper chamber and incubated in serum-free medium containing the indicated concentrations of SC. The serum was added to the medium of the lower chamber. After 24 h, the membrane was stained with hematoxylin and eosin (H&E). (A) Representative pictures were acquired using a microscope and (B) migrated cells were counted. The results are shown as the means  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to the control.

The results demonstrated that SC decreased the expression of CXCR4 and CXCR6 (Fig. 5A). In addition, the results from fluorescence microscopic observation revealed that SC suppressed actin filament rearrangement (Fig. 5B). These data suggest that the inhibitory effects of SC on the migratory activity of the Hep3B cells may be due to reduction in chemokine receptor expression and the suppression of actin filament polymerization.

*SC inhibits the phosphorylation of ERK and p38.* To determine which molecular signaling pathways are involved in the inhibitory effects of SC on the invasion and migration of Hep3B cells, the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs) was examined. The results from western blot analysis revealed that the phosphorylation levels of ERK and p38 MAPKs were decreased by SC treatment at the concentration of 50 and 100  $\mu$ g/ml. However, the phosphorylation level of JNK was slightly increased by SC treatment (Fig. 6A). In addition, treatment with SC did not affect on the protein levels of inhibitor of

$\kappa$ B (I $\kappa$ B) and the p65 subunit of NF- $\kappa$ B (Fig. 6B). These results suggested that the ERK and p38 signaling pathways may be involved in the expression of the molecules involved in the invasion and migration of Hep3B cells.

## Discussion

The process of cancer metastasis is composed of multiple sequential steps, such as invasion, intravasation, survival in the circulation, extravasation and growth in newly metastatic sites (3,4). As metastasis is one of most prevalence causes of cancer-related mortality, many researchers have aimed to develop drugs able to suppress the metastatic potential of cancer cells, including HCC (6,28). A number of natural products, such as baicalein from *Scutellaria baicalensis* (29), ardisinilioside from *Ardisia pusilla* (30), shikonin from *Lithospermum erythrorhizon* (31), acacetin from *Robinia pseudoacacia* (32) and caffeic acid phenethyl ester from propolis (33) have been reported to effectively inhibit the invasion and metastasis of HCC.



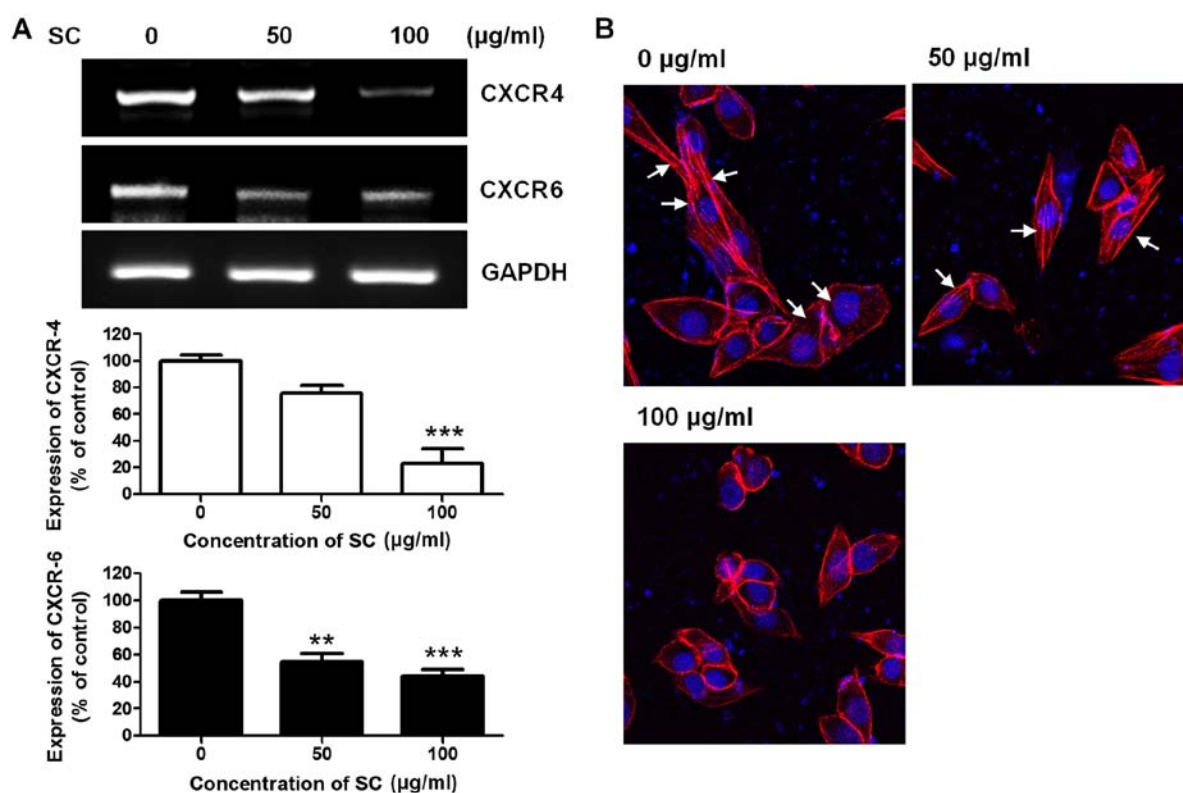


Figure 5. Inhibitory effect of *Sorbus commixta* (SC) on the expression of chemokine receptors and actin arrangement. (A) Hep3B cells were treated with the indicated concentrations of SC for 24 h. The expression levels of CXCR4 and CXCR6, were measured by RT-PCR. Densitometric results of the bands are presented as the means  $\pm$  SD of 3 independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to the control. (B) Hep3B cells were incubated in medium containing the indicated concentrations of SC for 24 h were fixed, stained with Texas Red-X phalloidin, and mounted with DAPI-containing solution. Images of stained cells were captured using a fluorescence microscope. Activated F-actin filaments are indicated by arrows.

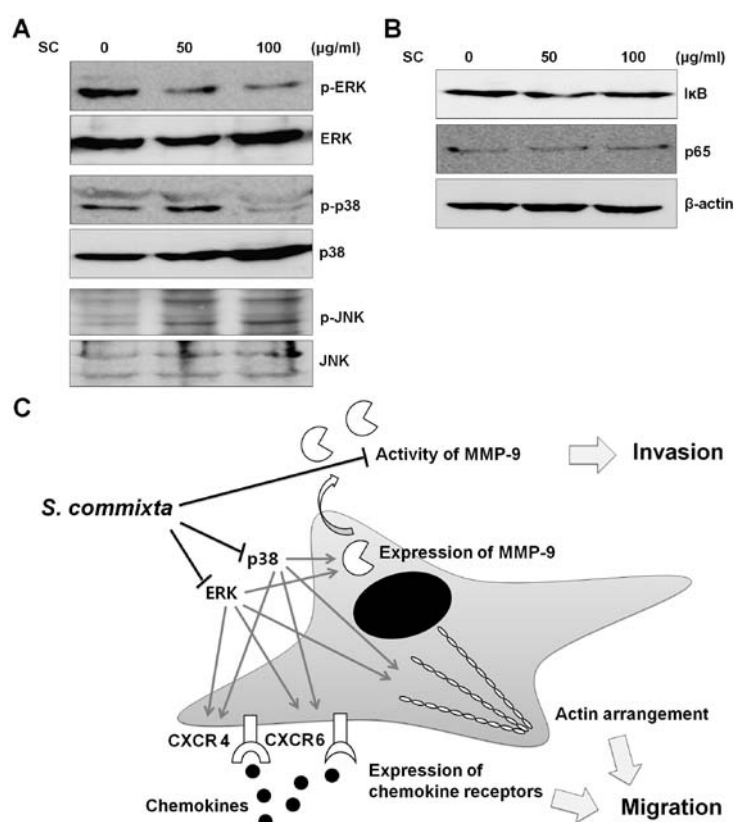


Figure 6. Inhibitory effect of *Sorbus commixta* (SC) on the activation of mitogen-activated protein kinase (MAPK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways. Hep3B cells were treated with the indicated concentrations of SC for 24 h. The activation of (A) MAPKs and (B) NF- $\kappa$ B pathways was examined by western blot analysis. (C) Schematic representation of the effect of SC on the invasion and migration of Hep3B cells and the underlying mechanism are presented.

MMP-9 is an enzyme which plays a role in the process of tumor metastasis, as it is expressed in many types of cancer and can degrade type IV collagen, a major component of the basement membrane (25,34). In addition, MMP-9 was recently reported as a modulator of signaling pathways that are involved in cell growth, migration and angiogenesis in a non-enzymatic manner (24,35). In our ongoing efforts to identify a novel MMP-9 inhibitor from medicinal herbs, we successfully identified the water extract of SC as an inhibitor of the enzymatic activity of MMP-9 using *in vitro* gelatin zymography (Fig. 2A). In addition, SC decreased the secretion and expression of MMP-9 in Hep3B cells at a non-toxic concentration (Fig. 2B, C and D). Furthermore, the results from Transwell invasion assay clearly indicated that SC inhibited the invasive potential of Hep3B cells (Fig. 3). As shown in our previous study (36), the knockdown of MMP-9 expression using siRNA was sufficient to reduce the invasive ability of Hep3B cells. These results suggest that SC has an inhibitory effect on the invasiveness of Hep3B cells by suppressing the expression and enzymatic activity of MMP-9.

We then further examined the effects of SC on the migration of Hep3B cells using Boyden chamber migration assay. As shown in Fig. 4, SC reduced the migratory ability of Hep3B cells. The cell surface receptors for chemokines highly expressed in various types of cancer, including HCC, such as CXCR4 and CXCR6, are known to play principle roles in cancer metastasis, particularly in the extravasation of tumor cells (26,27). Thus, in this study, we examined the expression of CXCR4 and CXCR6 in SC-treated HCC cells. The expression levels of CXCR4 and CXCR6 were decreased by SC treatment in a dose-dependent manner, as evaluated by RT-PCR (Fig. 5A). In previous studies, CXCR4 and CXCR6 have been reported as regulators of cytoskeleton arrangement (37-39). In addition, cytoskeleton arrangement is responsible for the motility of cancer cells (4). Therefore, in this study, we examined whether SC affects actin organization in Hep3B cells. The results shown in Fig. 5B demonstrate that SC markedly reduced stress fiber and lamellipodia formation responsible for the migration of cells.

In previous studies, the extract of SC was shown to act as an anti-inflammatory agent by blocking the nuclear translocation of NF- $\kappa$ B and its upstream pathways, such as phosphoinositide 3-kinase (PI3K) and Akt, in RAW 264.7 macrophage-like cells and vascular tissues of rats (11,14). The signaling pathways such as NF- $\kappa$ B and PI3K/Akt are also related with the expression of MMP-9 (33,34) and CXCR4 (40,41). In addition, PI3K/Akt is one of most essential pathways responsible for Rho-kinase activation and actin filament arrangement (42). Thus, we assumed that the inhibitory effects of SC on the expression of MMP-9 and CXCR4, and the arrangement of the actin cytoskeleton may be due to the suppression of the NF- $\kappa$ B and PI3K/Akt signaling pathways. However, our results clearly demonstrated that SC did not suppress the activation of NF- $\kappa$ B in Hep3B cells (Fig. 6B). This disagreement may be due to the different cell type or external stimuli used. On the other hand, SC inhibited the phosphorylation of ERK and p38, but not that of JNK (Fig. 6A). These signaling pathways, including p38 and ERK, are also involved in the expression of MMP-9 (34,43), CXCR4 and CXCR6 (44,45). In addition, previous studies have revealed that F-actin re-arrangement is also regulated by the p38 or ERK signaling pathways (46,47). Therefore, we suggest that the suppression of these molecules, which are involved in

migration of tumor cells, may be mainly regulated by the p38 and ERK pathways.

Furthermore, the methanol extract of SC has been shown to reduce the expression of adhesion molecules, including ICAM-1, VCAM-1 and E-selectin, in the aortas of rats with atherosclerosis induced by fructose (14). As these molecules are also responsible for the adhesion of cancer onto vascular epithelial cells (2,4), it is possible that SC inhibits the metastatic potential through the inhibition of these adhesion molecules. The metastasis of cancer is the main cause of cancer-associated mortality of most cancer patients (28). Therefore, the prevention of tumor metastasis has been the most important challenge in the development of anticancer drugs (48). Recently, many medicinal herbs and natural products have been discovered to have anti-metastatic activities and many researchers have focused on developing novel candidates for anti-metastatic agents (49,50).

In conclusion, in this study, and to the best of our knowledge, we are the first to demonstrate that SC suppresses the invasive and migratory potential of Hep3B cells through the inhibition of MMP-9, CXCR and cytoskeleton arrangement. The ERK and p38 signaling pathways may be involved in the inhibitory effects of SC on the invasive and migratory properties of Hep3B cells (Fig. 6C). From these results, we suggest that SC may be a potential candidate for drug development to inhibit tumor metastasis, particularly invasion, intravasation and the extravasation of cancer cells in multiple steps of cancer metastasis. Further extensive studies to determine the efficacy and safety of SC in *in vivo* experiments are required.

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